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ORIGINAL ARTICLE

Post-pollination mechanisms in *Nicotiana longiflora* and *N. plumbaginifolia*: pollen tube growth rate, offspring paternity and hybridization

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Abstract In natural populations where interfertile species coexist, conspecific and heterospecific pollen can be delivered to the stigmas. Post-pollination mechanisms might determine the seed siring success of different pollen donors within species as well as the chances for hybridization between species. *Nicotiana longiflora* and *N. plumbaginifolia* occur in sympatry in Northwest Argentina, where they have overlapping flowering seasons and share floral visitors. We explored (1) pollen tube growth rates for outcross versus self pollen in single-donor pollinations; (2) siring success of self versus outcross pollen donors in competitive pollinations, and (3) possibilities for hybridization by performing two- (outcross conspecific vs. heterospecific) and three-pollen donor (self vs. outcross vs. heterospecific) crosses. In *N. longiflora,* both pollen tube growth rate and siring success favored outcross pollen over self pollen and strong rejection of heterospecific pollen. In *N. plumbaginifolia,* pollen tube growth rate was similar for self and outcross pollen, self pollen sired similar numbers of offspring than outcross pollen and heterospecific pollen sired roughly the same number of progeny than self pollen. Results suggest that in natural sympatric populations, interspecific crosses would likely lead to unidirectional hybridization with *N. plumbaginifolia* as the seed parent.

Keywords Competitive pollinations · Cryptic self-incompatibility · Paternity success · Sympatry

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Introduction

In plants, post-pollination mechanisms can act as selective agents determining the siring success of pollen donors (Marshall and Ellstrand 1986; Marshall 1988, 1991; Snow and Spira 1991; Montalvo 1992; Walsh and Charlesworth 1992; Rigney et al. 1993; Carney et al. 1994; Marshall et al. 1996; Eckert and Allen 1997; Skogsmyr and Lankinen 1999; Marshall and Diggle 2001; Marshall and Oliveras 2001; Lankinen and Skogsmyr 2002; Shaner and Marshall 2003; Bernasconi et al. 2004; Haileselassie et al. 2005; Kruszewski and Galloway 2006; Marshall et al. 2007). For instance, self-incompatibility reactions are a common post-pollination mechanism present in angiosperms (de Nettancourt 2001; Franklin-Tong and Franklin 2003). Also, the preference of some plants for outcross pollen over self pollen when growing together in the style but being able to self-fertilize when no other sources of pollen are present (i.e. cryptic self incompatibility) is a common post-pollination phenomenon (Bateman 1956; Bowman 1987; Casper et al. 1988; Hessing 1989; Weller and Ornduff 1989; Aizen et al. 1990; Cruzan and Barrett 1993; Rigney et al. 1993; Travers and Mazer 2000; Kruszewski and Galloway 2006). Many other pre- and post-zygotic phenomena may occur in the pistils and thus determine the siring success of different pollen genotypes (e.g., variation in maternal abortion or seed provisioning, hybrid vigor).

In addition, intrinsic traits to each species, such as pollen tube growth rate and style length might also have an impact on the siring success of pollen donors. For example, it is often the case that pollen tube growth rate is correlated with pollen size and/or style length (Aizen et al. 1990; Williams and Rouse 1990; Diaz and Macnair 1999; Lee et al. 2008). Thus, style length becomes of particular importance when heterospecific pollen from species with contrasting style length is delivered to the stigma (e.g., Diaz and Macnair 1999; Lee et al. 2008). Because pollen tubes from long-styled species must typically grow for longer distances than pollen tubes from short-styled species, pollen tubes from long-style species may have an advantage in fertilizing ovules of a short-style species. Thus, whenever there is interspecific pollen transfer between long- and short-styled relatives, asymmetric hybridization may be common (Emms et al. 1996; Lee et al. 2008).

Post-pollination selective mechanisms can determine the siring success of pollen donors and may prevent hybridization between sympatric species, even with overlapping flowering seasons. Although interspecific pollen transfer is a prerequisite to hybridization, few studies have been carried out to study its outcome and consequences (Carney et al. 1994; Emms et al. 1996; Diaz and Macnair 1999). In particular, there is a lack of literature investigating the importance of post-pollination mechanisms as isolation barriers between naturally crossable species with contrasting floral morphology and mating systems, such as our study species.

Nicotiana longiflora and *N. plumbaginifolia* are self-compatible, sister species that occur in sympatry in northern Argentina (Goodspeed 1954). Because these species share pollinators and have overlapping flowering seasons (Figueroa-Castro 2008), it is possible that the delivery of pollen from different sources (self, conspecific outcross, and interspecific) might occur. *N. longiflora* has a long style (73.905 mm ± s.e. 5.195) and is a strong outcrosser, whereas *N. plumbaginifolia* has a short-style (25.043 mm ± 1.264) and high levels of self-fertilization (Figueroa-Castro 2008.). Therefore, if intraspecific pollen interactions occur within these species, cryptic SI will be expected for *N. longiflora* but not for *N. plumbaginifolia*. In the case of mixed pollinations in which not only self and outcross, but also interspecific-pollen is delivered on the stigma, pollen from the long-styled *N. longiflora* might have an advantage over *N. plumbaginifolia*'s pollen because *N. plumbaginifolia*'s pollen tubes are not able to grow as far as *N. longiflora*'s pollen tubes.

The main objective of this study is to determine the siring success of different pollen sources (self, conspecific outcross and interspecific) of *N. longiflora* and *N. plumbaginifolia* when delivered on conspecific and heterospecific stigmas. The specific goals are to quantify: (1) differences in pollen tube growth rate between self and outcross pollen for each species; (2) intraspecific bias in siring success of outcross versus self pollen donors, and (3) probability of hybridization when two (outcross vs. heterospecific pollen) and three (self vs. outcross vs. heterospecific pollen) sources of pollen interact in the styles of each species.

Methods

Study species and plant material

Nicotiana longiflora and *N. plumbaginifolia* (Solanaceae) are each other's closest relatives (Ippolito 2000; Chase et al. 2003; Clarkson et al. 2004; Lim et al. 2006). These species are self-compatible and crossable in the greenhouse (East 1916; Goodspeed 1954; Lee et al. 2008). Flowers of *N. longiflora* are 40–120 mm in corolla length (Goodspeed 1954) and the species has been confirmed as a strong outcrosser (Figueroa-Castro 2008). On the other hand, *N. plumbaginifolia* has shorter flowers (25–35 mm in length; Goodspeed 1954) and it has been suggested to have a mixed mating system (Figueroa-Castro 2008). These species occur in sympatry in northern Argentina. From there, *N. longiflora* is distributed to the south and southeast of the continent, whereas *N. plumbaginifolia's* range extends to the North of the continent and into Mexico (Goodspeed 1954). The co-occurrence of the two species in sympatric populations and the overlap in their flowering seasons was shown to have a negative effect on *N. longiflora* seed set and *N. plumbaginifolia* outcrossing rates (Figueroa-Castro 2008).

Plants used in this study were grown from seeds collected in natural populations. Naturally pollinated seeds were collected in 2005 from two natural populations [Mango (S 23°48′36″, W 64°47′46″; 492 m altitude) and Canal (S 23°46′36.8″, W 64°49′42.6″; 544 m altitude)], in the Province of Jujuy in northern Argentina, where both *Nicotiana* species co-occur in sympatry.

Pollen tube growth rate

The rate of growth of pollen tubes was determined for each species through single-donor hand-pollinations conducted in the greenhouse. Four flowers per plant were emasculated within 24 h before anthesis and anther dehiscence and then hand-pollinated on the first day of anthesis. Single-donor pollinations were done by rubbing two to three recently dehiscent anthers on the stigmas of plants grown in the greenhouse. One of two sources of pollen was applied: (1) self pollen, collected from other flowers on the same plant, and (2) outcross pollen,

collected from 2 to 3 other plants within the same population. Flowers pollinated with each kind of pollen were collected at 12, 24, 36, and 48 h from the time of pollination. Styles were fixed for at least 1 h in 3:1 ethanol: acetic acid, cleared and softened by autoclaving for 15 min in 10% (w/v) sodium sulfite solution, stained for at least 12 h in 0.1 M tripotassium phosphate, 0.1% (w/v) aniline blue solution and squashed (Kho and Baer 1968). Pollen-tube length was measured under a Leica MZFLIII stereoscope fitted with a UV filter, provided by the Electron Microscopy core facility at the University of Missouri-Columbia. Pollen tube length was measured from the tip of the stigma to where ten pollen tubes were visible; the leading edge of the large mass of pollen tubes.

A total of 160 flowers per species were used for this experiment [20 flowers × 2 sources of pollen (self and outcross) × 4 collection times]. Flowers were chosen according to the availability of new flowers per plant, prior to anthesis and anther dehiscence. Each kind of pollen source (self and outcross) was applied to 80 flowers over 20 *N. longiflora* and 40 *N. plumbaginifolia* pistil parents. Because flower availability was limited in *N. longiflora*, some plants had two sets of treatments.

Statistical analyses: pollen tube growth

Pollen tube growth rate was estimated as the distance traveled by pollen tubes divided by the time styles were collected. Therefore, pollen tube growth rate was calculated for the four periods of time styles were collected. Pollen tube growth per unit of time was analyzed in SAS 9.1 with an analysis of covariance using species, time of style collection (12, 24, 36 and 48 h after pollination) and treatment (self vs. outcross pollen) as independent variables, style length as a covariate and pollen tube growth rate as the dependent variable. All interactions were also included in the model (species × time of style collection; species × treatment; time of style collection × treatment; species × time of style collection × treatment). Post hoc Tukey tests were conducted to determine pairwise differences among groups.

Mixed pollinations and paternity determination

Plant material

All crosses for the evaluation of paternity from mixed pollinations were conducted on plants grown in the controlled environment of the greenhouse (14 h days at 24°C and 10 h nights at 13°C at the University of Missouri-Columbia). Seeds were rinsed with 70% bleach and then twice with sterilized water. Seeds were planted with 500 µl of 750 ppm gibberellin solution in trays with pre-mixed soil and covered with vermiculite. Plants were watered every other day.

Pollen quantification

In order to conduct assays with pollen mixtures to investigate the chances of hybridization between the *Nicotiana* species, we first quantified the average number of pollen grains per anther per species. Six to eight plants per population and species were chosen for pollen quantification. From each plant, four or five mature but indehiscent anthers from at least two flowers per plant were collected for pollen quantification (*N. longiflora*: 30 and 39 anthers for Mango and Canal, respectively; *N. plumbaginifolia*: 34 and 32 anthers for Mango and Canal, respectively). Anthers were left to dehisce and dry in uncapped 1.5 ml microcentrifuge tubes stored in a dust-free space. Pollen number was estimated with an Elzone 280 PC particle counter, previously calibrated with 40 µm beads, which are in the range of pollen size for both *Nicotiana* species (15–45 µm). 1.5 ml of 2% saline solution were added to each tube, anthers were dissected with forceps and sonicated for 4 min. Tubes were then rinsed into a 20 ml sample vial which was left from 8 h to overnight to settle. Pollen estimations were done after sonicating each 20 ml vial for 1 min. Five counts were made for each tube, the highest and lowest scores were discarded and the three other counts were used to get the average number of pollen grains per anther. The average number of pollen grains per anther indicated that the ratio of *N. longiflora* (29322.49 ± s.e. 2035.97; 95% CI: 25332.05–33312.93; $N = 69$ anthers from 13 plants) to *N. plumbaginifolia* (7738.59 ± 543.37, 95% CI: 6673.61–8803.57; $N = 66$ anthers from 14 plants) pollen per anther is 3.8:1. Therefore, we used four *N. plumbaginifolia* anthers and one *N. longiflora* anther in an attempt to apply approximately a 1:1 ratio pollen number in interspecific crosses (described below).

Two- and three-donor crosses

Two-donor crosses were applied on plants from both Canal and Mango populations, whereas three-donor crosses were conducted only on plants from the Mango population. Three-donor crosses were not applied on plants from the Canal population because there were not enough plants available with different genotypes to determine seedling paternity. To quantify the paternity results from mixed pollinations, plants that were homozygous for alternative alleles were chosen. For three-donor crosses two markers were used. One marker was used to distinguish between self and outcross fertilizations within species (Cos 16 for *N. longiflora* and NA6 for *N. plumbaginifolia*; see the offspring genotyping below for details) and the other

marker was used to distinguish between intra- and interspecific fertilizations. For all crosses we used six maternal plants per species per population. In all cases those six plants also acted as pollen donors within species (self and/or outcross pollen donors; Fig. 1). Each maternal plant was crossed with three different pollen donors, for a total of 18 crosses per species per population. For outcross intraspecific versus interspecific pollen crosses, all six maternal plants acted as outcross pollen donors, whereas three plants from the other species were used as donors of interspecific pollen (Fig. 1b). Three-donor crosses were conducted in a similar way, the six maternal plants also acted as self and outcross pollen donors and three plants form the other species were used as pollen donors of interspecific pollen (Fig. 1c). In those cases in which the plants originally selected for crosses did not have dehiscent anthers, other plants with the same genotype were used. Thus, final sample sizes were five to eight maternal plants per cross type, per two populations, per two species.

(a) Self vs. outcross pollen

Maternal plant	*Pollen donors*		
A	A+D	A+E	A+F
B	B+D	B+E	B+F
C	C+D	C+E	C+F
D	D+A	D+B	D+C
E	E+A	E+B	E+C
F	F+A	F+B	F+C

(b) Outcross vs. interspecific pollen

Maternal plant	*Pollen donors*		
A	D+1	E+2	F+3
B	D+2	E+3	F+1
C	D+3	E+1	F+2
D	A+1	B+2	C+3
E	A+2	B+3	C+1
F	A+3	B+1	C+2

(c) Self vs. outcross vs. interspecific pollen

Maternal plant	*Pollen donors*		
A	A+D+1	A+E+2	A+F+3
B	B+D+2	B+E+3	B+F+1
C	C+D+3	C+E+1	C+F+2
D	D+A+1	D+B+2	D+C+3
E	E+A+2	E+B+3	E+C+1
F	F+A+3	F+B+1	F+C+2

Fig. 1 Crossing design to test for pollen tube interactions in *N. longiflora* and *N. plumbaginifolia* via paternity determination. Each cell shows the combinations of pollen donors used for (**a**) self versus outcross mixed pollinations, (**b**) outcross versus interspecies mixed pollinations, and (**c**) self versus outcross versus interspecies mixed pollinations. *Letters* indicate pollen donors within the species, whereas *numbers* correspond to interspecies pollen donors

Pollen from recently dehisced anthers from each parent was mixed thoroughly in 0.5 ml microcentrifuge tubes and applied with flat toothpicks on stigmas of flowers emasculated within 24 h before anthesis. In self versus outcross pollen crosses the same number of anthers from each donor were used (2–3 anthers per parent); whereas in interspecific crosses four *N. plumbaginifolia* anthers per each *N. longiflora* anther were used to approximate a 1:1 ratio of pollen grains. Pollinations were conducted within 24 h after anthesis. Abundant pollen was applied to all stigmas such that a layer of pollen was clearly visible and thus favoring that pollen load vastly exceeded the number of ovules. Mature fruits were collected before seed dispersal (2 and 3 weeks for *N. plumbaginifolia* and *N. longiflora*, respectively) and used to determine seedling paternity.

Offspring genotyping

Seeds from each kind of cross were planted and grown in the greenhouse as previously described (plant material section above). A total of 360 seedlings per cross per species were grown for genotyping (10 seedlings × 18 crosses × 2 populations). Because some of the crosses did not set any seeds and in some cases seeds did not germinate or seedlings died before being large enough to extract DNA, the number of seedlings genotyped was reduced to 24 *N. longiflora* and 62 *N. plumbaginifolia* maternal parents for self versus outcross pollen crosses; 286 *N. longiflora* and 230 *N. plumbaginifolia* for outcross versus interspecific-crosses and 124 *N. longiflora* and 62 *N. plumbaginifolia* for three-donor pollinations. This reduction in sample sizes hardly had an effect on the significance of our results (Fig. 3, Table 2).

DNA was extracted from leaves as soon as seedlings had enough vegetative tissue (around 1 month after seed planting). Tissue was ground in 300 µl extraction buffer (0.35 M Sorbitol, 0.1 M Tris pH 7.5, 5 mM EDTA) with β-mercaptoethanol, 300 µl lysis buffer and 100 µl sarkosyl; then incubated for 10 min at 65°C; extracted twice with 24 chloroform:1 isoamyl alcohol; rinsed with 76% EtOH-10 mM $NH_4C_2H_3O_2$ and stored in 1/10 TE/RNase.

Seedlings were genotyped using the codominant CAPS markers cellulose synthase D-like protein mRNA (accession number AF304375; locus abbreviation, NA6) and tRNA methyltransferase (accession number AT2G45730; locus abbreviation, COS-16). Both of these markers are polymorphic for *N. longiflora* (COS 16 alleles: 950 bp and 800 + 150 bp; NA6 alleles: 900 bp and 500 + 400 bp).

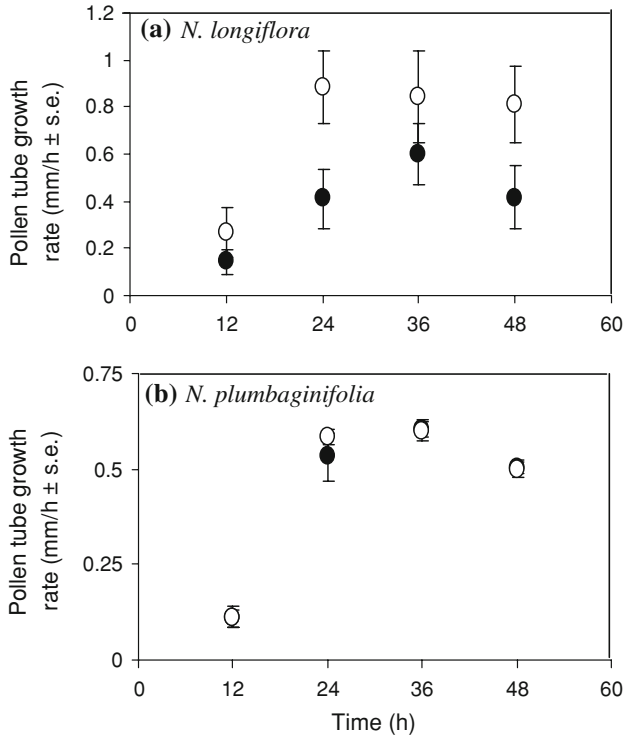

Fig. 2 Changes in pollen tube growth rate (±s.e.) of outcross (*open circles*) and self (*closed circles*) pollen tubes growing in conspecific styles of *N. longiflora* (**a**) and *N. plumbaginifolia* (**b**). Post hoc Tukey tests showed that self pollen tube growth rate was significantly lower than outcross pollen in *N. longiflora* styles

On the other hand, *N. plumbaginifolia* was polymorphic for the NA6 marker (alleles: 500 + 400 bp and 500 + 220 + 180 bp) but not for the COS16 marker (i.e. single band at 900 bp).

DNA was amplified for COS-16 (39 cycles with 2.75 min at 94°C; 1 min at 55°C, 1 min at 72°C) and NA6 (39 cycles with 2.75 min at 94°C; 1 min at 60°C, 1.5 min at 72°C) and digested for 2 h to overnight with *Eco*RI and *Hha*I, respectively. Digested products were run on 1.5% agarose gels at 70 V for 2.5 h and visualized with UV light.

Statistical analyses: paternity from two- and three-donor crosses

We used χ^2 tests to determine if the number of seeds sired by different pollen sources was nonrandom, that is, as expected based on the pollen mixtures applied on the stigmas; data were analyzed with several χ^2 tests. First, we tested if offspring sired by different sources of pollen (self vs. outcross; outcross vs. interspecific; and self vs. outcross vs. interspecific) was significantly different from the expected ratios of 1:1 or 1:1:1, for the two- and three-donor pollinations, respectively. Because pollen mixtures might not be exactly in a 1:1 ratio, a set of tests with expectations 0.55:0.45 and 0.6:0.4 were also performed on data of two-donor crosses. We performed these tests as a way to assess the robustness of our pollen mixtures. Therefore, the 0.55:0.45 and 0.6:0.4 expectations tested the validity of our findings even if our pollen mixtures were 10–20% biased. Because several tests were applied to the same set of data, significance was considered at $P = 0.01$ in order to avoid Type I error.

We also used the CATMOD procedure in SAS to test the effect of species, population and their interaction in the number of seeds produced by each type of pollen in the self versus outcross and outcross versus interspecific crosses. Similarly, we also tested the effect of species in the number of seeds produced by each type of pollen in the three-donor crosses. Because in the outcross versus interspecific cross one of the cells was a zero (i.e. not seeds were obtained by interspecific pollen grains on *N. longiflora* from the Canal population), there was a constraint on degrees of freedom, and therefore, the interaction species × population was not included in the model.

Results

Pollen tube growth rate in selfed versus outcrossed single-donor pollinations

Analysis of covariance showed a significant effect of pollination treatment, time of style collection, and the interaction pollen treatment × species on pollen tube growth rate (Fig. 2, Table 1). Pollen tube growth rate was not significantly affected by species or the interactions species × time, time × treatment and species × time × treatment (Table 1). Overall, pollen tube growth rate was significantly lower at 12 h than at any other time of style collection (Fig. 2). In *N. longiflora*, outcross pollen showed a significantly higher growth rate (0.70 ± 0.08 mm/h) than self pollen (0.39 ± 0.06 mm/h; Fig. 2a, Table 1). However, growth rates of outcross (0.56 ± 0.03 mm/h) and self pollen (0.54 ± 0.03 mm/h) were indistinguishable in *N. plumbaginifolia* (Fig. 2b, Table 1). Mean pollen tube growth rate for *N. plumbaginifolia* was 0.55 ± 0.02 mm/h whereas for *N. longiflora* it was 0.54 ± 0.06 mm/h.

Offspring paternity from self and outcross competitive pollinations

The observed proportions of selfed versus outcrossed seeds in mixed pollinations were significantly different from 1:1 expectations (Table 2); *N. longiflora* set almost 15 times (306 out of 327) more outcrossed seeds than self seeds

Table 1 Analysis of covariance to test for differences in self and outcross pollen tube growth rate between *Nicotiana longiflora* and *N. plumbaginifolia* at different times of style collection. Style length was included as covariate

Source	*df*	Mean squares	*F*	*P*
Style length	1	0.050	0.49	0.484
Species	1	0.051	0.50	0.482
Style collection time	3	1.619	15.84	<0.0001
Pollination treatment	1	1.128	11.04	0.0011
Species × style collection time	3	0.045	0.44	0.725
Species × pollination treatment	1	0.815	7.98	0.005
Style collection time × pollination treatment	3	0.074	0.73	0.537
Species × style collection time × pollination treatment	3	0.051	0.50	0.681

Table 2 χ^2 tests performed to determine offspring paternity, in two-donor (self vs. outcross and outcross vs. interspecies) and three-donor (self vs. outcross vs. interspecies) mixed pollinations in *Nicotiana longiflora* (A) and *N. plumbaginifolia* (B)

	Self versus outcross	Outcross versus interspecies	Self versus outcross versus interspecies
Nicotiana longiflora			
1:1 expectation	248.4***	258.7***	61.5***
0.55:0.45 expectation	196.6***	209.2***	–
0.6:0.4 expectation	153.2***	168.0***	–
Nicotiana plumbaginifolia			
1:1 expectation	16.0***	167.0***	22.9***
0.55:0.45 expectation	5.4 ns	131.4***	–
0.6:0.4 expectation	0.4 ns	101.9***	–

Tests of offspring paternity within species were conducted for a 1:1 expectation and biased expectations of 0.55:0.45 and 0.6:0.4 in two-donor crosses and 1:1:1 expectations in three-donor crosses. Because multiple tests were conducted with the same data, significance was considered at $P = 0.01$. *** $P \ll 0.001$, ns $= P > 0.01$

(Fig. 3a, Table 2). *N. plumbaginifolia* showed a smaller bias, but in the opposite direction, tending to set more selfed seed (Fig. 3a; Table 2). The proportions of outcross (110 out of 288) and self offspring (178 out of 288) were only slightly different from a 1:1 ratio (Fig. 3a, Table 2). Also, the proportions of self versus outcrossed seeds was significantly different between species, but not between populations nor was it affected by the interaction species x population (Table 3).

Because we did not count pollen applied to stigmas directly, relying instead on average pollen number per anther per species, we also tested the sensitivity of these tests to sampling variation in pollen ratios applied to stigmas. Even if the pollen ratios applied had been consistently off by as much as 20%, the seed set bias in *N. longiflora* remained significant (Fig. 3a, Table 2). In *N. plumbaginifolia*, the bias toward setting self seeds was not significant if self to outcross pollen ratios had consistently been 10–20% off (0.55:0.45 and 0.6:0.4 expectations, respectively; Table 2). The conservative interpretation here is that in *N. plumbaginifolia,* there is little or no bias toward setting selfed versus outcrossed seed.

Offspring paternity from outcross versus interspecific pollinations

Results for outcross versus interspecific offspring paternity from mixed pollinations showed a significant departure from the 1:1 ratio expected based on pollen mixtures for both *N. longiflora* and *N. plumbaginifolia* (Fig. 3b, Table 2). The frequency of offspring from each kind of pollen was also significant for both species when testing the observed frequencies against the expected 0.55:0.45 and 0.6:0.4 ratios (Fig. 3b; Table 2).

Nicotiana longiflora and *N. plumbaginifolia* showed contrasting patterns in the number of outcross versus hybrid offspring between species (Table 3). Around 40 times more conspecific outcross *N. longiflora*'s seeds were sired (279 out of 286) than interspecific seeds (7 out of 286) (Fig. 3b). In contrast, seeds in *N. plumbaginifolia* were more successfully sired by interspecific pollen (213 out of 230) (Fig. 3b; Table 2). These results hold even if our pollen ratios were off by as much as 20%. The siring success of outcross versus interspecific pollen grains was not significantly different between populations (Table 3).

Three-donor pollinations

The observed frequencies of self, outcross, and hybrid offspring from each species was significantly different from the expected 1:1:1 ratio for both *N. longiflora* and *N. plumbaginifolia* (Fig. 3c, Table 2). Moreover, the number of seeds sired by each kind of pollen was significantly different between species (Fig. 3c, Table 3).

In *N. longiflora* most offspring were produced via conspecific outcross fertilizations (81 out of 124), whereas in *N. plumbaginifolia* most offspring were produced by self or interspecific pollen (28 and 31 out of 62, respectively). Seeds from outcross, conspecific donors were underrepresented in *N. plumbaginifolia* (Fig. 3c).

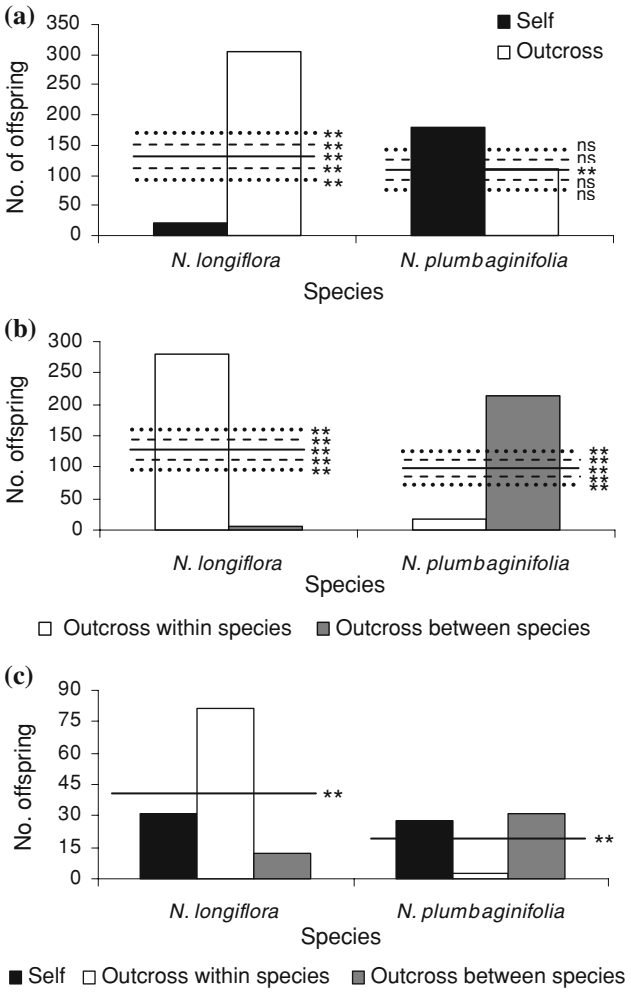

Fig. 3 Offspring obtained through two- and three-donor mixed pollinations in *N. longiflora* and *N. plumbaginifolia*. **a** Number of offspring sired by self and outcross mixed pollinations. **b** Number of offspring sired by intra and interspecific mixed pollinations. **c** Number of offspring sired by self, outcross and interspecific mixed pollinations. Expected frequencies under random mating (1:1 and 1:1:1 ratio; *solid line*) and under the biased expectations of 0.55:0.45 (*dashed lines*) and 0.6:0.4 (*dotted lines*) are shown. *Asterisks* indicate significant differences between observed and expected frequencies at $P < 0.01$. ns = not significant differences between observed and expected frequencies

Table 3 Maximum likelihood ANOVA (CATMOD procedure of SAS) for number of seeds of *N. longiflora* and *N. plumbaginifolia* produced by different sources of pollen in two- and three-donor mixed pollinations: (a) self versus outcross pollen mixtures; (b) outcross versus interspecific pollen mixtures and, (c) self versus outcross versus interspecific pollen mixtures

Source	*df*	χ^2	*P*
(a) Self versus outcross pollen			
Intercept	1	73.56	<0.0001
Species	1	151.53	<0.0001
Population	1	0.25	0.6161
Species X population	1	0.08	0.7808
(b) Outcross versus interspecific pollen			
Intercept	1	6.34	0.012
Species	1	179.37	<0.0001
Population	1	0.54	0.463
(c) Self versus outcross versus interspecific pollen			
Intercept	2	6.28	0.0432
Species	2	39.08	<0.0001

The interaction species × population in the outcross versus interspecific pollen cross was not included in the model because it was constrained by degrees of freedom (see text for more details)

Discussion

In this study, we have demonstrated that post-pollination mechanisms occur in both *Nicotiana* species. In *N. longiflora,* both pollen tube growth rate and siring success favored outcross pollen over self pollen and strongly rejected heterospecific pollen. In *N. plumbaginifolia,* pollen tube growth rate was similar for self and outcross pollen, self pollen sired similar numbers of offspring than outcross pollen and heterospecific pollen sired roughly the same number of progeny than self pollen. Our results suggest that post-pollination mechanisms are common in *N. longiflora* and may be of great relevance in sympatric populations. Thus, in natural sympatric populations interspecific crosses between these species may occur and; as a consequence of post-pollination mechanisms, unidirectional hybridization with *N. plumbaginifolia* as the seed parent would likely take place.

Pollen tube growth rate and self versus outcross paternity success

In self-compatible species, differential pollen tube growth rate of self and outcross pollen grains may function as a post-pollination mechanism that favors outcross in self + outcross pollen mixtures (i.e. cryptic self-incompatibility; Bateman 1956; Bowman 1987; Casper et al. 1988; Hessing 1989; Weller and Ornduff 1989; Aizen et al. 1990; Cruzan and Barrett 1993; Rigney et al. 1993; Travers and Mazer 2000; Kruszewski and Galloway 2006). Here, we found that *N. longiflora* has a similar post-pollination bias, so that outcross pollen grains have a significant fertilization advantage. This phenomenon has also been recorded in other self-compatible species with floral traits typical of outcrossing mating systems (Bowman 1987; Cruzan and Barrett 1993; Jones 1994).

Our results showed that in *N. longiflora*, outcross pollen grains grow twice as fast as self pollen grains.

However, the number of seeds sired by outcross pollen grains was 15 times greater than the number of self seeds; suggesting that pollen tube growth rate is not a reliable predictor of siring success, such as has been documented in other studies (Marshall 1991; Marshall and Diggle 2001). Therefore, other mechanisms besides differential pollen tube growth rate favored the greater success of outcross grains at siring seeds. For instance, pre-zygotic mechanisms such as interactions between the style and pollen grains when growing through it may favor or prevent germination rate, and tube attrition, and thus inhibit or facilitate pollen tube growth (Bowman 1987; Casper et al. 1988; Hessing 1989; Weller and Ornduff 1989; Aizen et al. 1990; Cruzan 1990; Walsh and Charlesworth 1992; Cruzan and Barrett 1993; Jones 1994; Erbar 2003; Kruszewski and Galloway 2006). Therefore, this kind of phenomena may have favored the siring success of outcross pollen donors in *N. longiflora*. Moreover, post-zygotic mechanisms, such as seed abortion, fruit abortion, and seed filling may also favor outcross pollen grains (Charlesworth 1988; Weller and Ornduff 1991; Montalvo 1992; Eckert and Allen 1997; Erbar 2003). It is probable that both differential pollen tube growth rate between self and outcross pollen and selective abortion of seeds are favoring the high reproductive success of outcross pollen in *N. longiflora*. This is not surprising given the importance of pollinators for plant reproductive success, high outcrossing rates and low fixation indices previously recorded in *N. longiflora* (Figueroa-Castro 2008).

On the other hand, pollen tube growth rate of outcross and self pollen in *N. plumbaginifolia* was not significantly different, indicating that both sources of pollen have similar probabilities for ovule fertilization. This was supported with paternity determination of self versus outcross mixed pollinations. However, single locus outcrossing rates and fixation indices estimates from naturally grown *N. plumbaginifolia* plants showed that, at least in sympatric populations, this species is a strong selfer (Figueroa-Castro 2008). Moreover, *N. plumbaginifolia*'s floral morphology (short corolla tubes, and reduced herkogamy) and mating system is that of a strong selfer (Soule 2007; Figueroa-Castro 2008). Thus, selection for competitive pollen is not expected to be strong since most pollen is selfed. Hence, outcross and self pollen have the same competitive ability, as estimated by pollen tube growth rate. Lack of differences in pollen tube growth rate between outcross and self pollen has also been found in other species (Casper 1985; Bertin and Sullivan 1988; Fenster and Sork 1988; Weller and Ornduff 1991; Travers and Mazer 2000), including species exhibiting a mixed mating system (Montalvo 1992; Baker and Shore 1995; Figueroa-Castro 2008).

Paternity success on two- and three-donor crosses with heterospecific pollen in *N. longiflora*

Nicotiana longiflora's conspecific outcross pollen was significantly more successful at siring seeds than self or interspecific pollen. The higher success of outcross pollen compared to self pollen at siring seeds might be a consequence of pollen tube growth rate being greater for outcross pollen than self pollen. The low success of interspecific pollen at siring seeds could also be related to differential pollen tube growth rates. Although pollen tube growth rates from *N. longiflora* and *N. plumbaginifolia* were not significantly different from each other, differences in growth rates for pollen from different sources are evident. The mean pollen tube growth rate of *N. longiflora*'s outcross pollen (0.7 ± 0.08 mm/h) was the greatest, compared with self pollen (0.39 ± 0.06 mm/h) or *N. plumbaginifolia*'s pollen (0.55 ± 0.02 mm/h). This difference may explain, at least partially the greater success of outcross pollen at siring seeds in *N. longiflora*.

However, other pre- and post-zygotic mechanisms may be operating too. In single pollen donor crosses, Lee et al. (2008) found that only pollen from species with similar style length was able to fertilize *N. longiflora*'s ovules. In single- and two-donor assays in other species, Emms et al. (1996) and Carney et al. (1994), also found a strong discrimination against heterospecific pollen.

Pollinator observations in the sympatric Mango population showed that only 3.7% of pollinator movements are interspecific (Figueroa-Castro 2008). Therefore, the 1:1 pollen proportions used in the current study probably have an excess of heterospecific pollen compared with what occurs in natural populations. Even though our pollen mixtures were not an exact representation of those that are probably found in sympatric natural populations, our study is unique being, to our knowledge, the first one conducting three-donor pollinations on single stigmas to test the paternity success of pollen from self, outcross, and interspecific sources.

Paternity success on two- and three-donor crosses with heterospecific pollen in *N. plumbaginifolia*

When *N. plumbaginifolia* acted as the maternal parent, hybridization and self-fertilization were strongly favored. In outcross versus interspecific crosses, significantly more seeds were sired by heterospecific pollen grains, probably as a consequence of *N. longiflora's* higher pollen tube growth rate (0.7 ± 0.08 mm/h vs. 0.56 ± 0.03 mm/h for outcross *N. plumbaginifolia* pollen). While we did not estimate pollen tube growth rate in interspecific crosses, several studies using interspecific crosses have shown that the success in fertilizing ovules is predicted by the style

lengths of the species being crossed (e.g., Aizen et al. 1990; Williams and Rouse 1990; Diaz and Macnair 1999), including our study species (Lee et al. 2008).

Our results showed that self and outcross pollen grains have similar pollen tube growth rate in *N. plumbaginifolia*, therefore in two- and three-donor crosses both sources of pollen were expected to have similar siring success. This was not true for three-donor crosses, in which both self and interspecific pollen grains had similar success at siring seeds and outcross pollen had the lowest siring success. This suggests that pollen tube growth rate may not be the only mechanism determining the siring success of pollen donors in *N. plumbaginifolia*. For instance, it is possible that interactions pollen-style may be selecting against outcross pollen grains and favoring self and interspecific pollen to fertilize the ovules. It has been suggested that in species with a long history of selfing, pollen has been selected to grow in particular stylar environments, thus conferring it with a slight competitive ability over outcross pollen (Cruzan 1990). Alternatively, the stylar tissue could have negative interactions with outcross pollen (Cruzan 1989). Moreover, the delivery of mixed pollen loads on the stigmatic surface of plants could lead to gametophytic selection (Marshall and Ellstrand 1985). For instance, pollen with the ability to germinate, grow and fertilize faster will be favored by selection. Further studies are needed in order to clarify pollen–pollen and pollen-stylar tissue interactions occurring in three-donor pollinations.

Implications

Results from crosses with mixed conspecific and heterospecific pollen demonstrated that hybridization between these *Nicotiana* species may be occurring in natural populations, especially in *N. plumbaginifolia*. Because in natural populations pollinators have a strong preference for *N. longiflora* over *N. plumbaginifolia* and because they may visit hybrids and parental species indiscriminately (Wendt et al. 2001; Ippolito et al. 2004); asymmetric hybridization and introgression between these species may be occurring, such that more *N. plumbaginifolia* genes are introduced into *N. longiflora* than in the reverse direction; such as has been documented for other species (Broyles 2002; Martin and Willis 2007). Therefore, further studies should address how much introgression has occurred and how much of the original genome from each species is still conserved. New studies using a higher number of polymorphic markers on populations outside the sympatric zone will be of great relevance to follow-up on these questions.

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